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DEVELOPMENT OF THE MARKINGS ON THE PUPAL WING OF *PIERIS RAPAE* (PIERIDAE)

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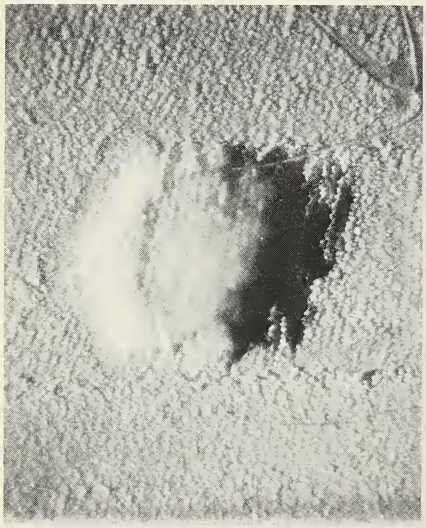
INTRODUCTION

The development of the black markings of *Pieris brassicae* (L.) was studied by Onslow (1916), who reported that the pupal wing became black all over when soaked in tyrosine solution while selective darkening of the markings occurred in tyrosinase solution. (The action of an oxidase, eg. tyrosinase, on a colorless chromogen, e.g. tyrosine, is known to give melanin pigments.) The conclusion was that the chromogen first was deposited in the areas destined to become black, then oxidase was supplied by the hemolymph, and finally atmospheric oxygen reached the surface of the wing and caused darkening where chromogen was concentrated. This explanation was modeled after that of Gortner (1911a) for development of the color pattern on the elytra of the Colorado potato beetle.

In contrast to Onslow's result, Braun (1939) found that "artificial pigmentation", i.e. selective darkening of the markings, occurred when pupal wings of *Papilio ajax* (L.) and *Ephestia kühniella* (Zeller) were soaked in tyrosine solution. Braun's explanation was that the darkened scales were less chitinized due to slower development and so were able to absorb the tyrosine solution. His conclusion was that oxidase is present in the scales in general, but at the "certain time of development pigment is present in the body" only the soft, less chitinized scales are able to accept this "pigment" (chromogen).

The general problem of development of the wing pattern in Lepidoptera has received study by Kühn, Goldschmidt, Köhler, and others, as reviewed in detail by Caspari (1941). Brief summaries are given by Wigglesworth (1965: 78) and Bodenstein (1953).



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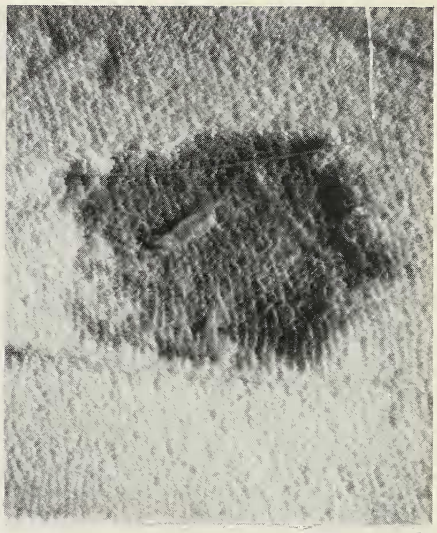

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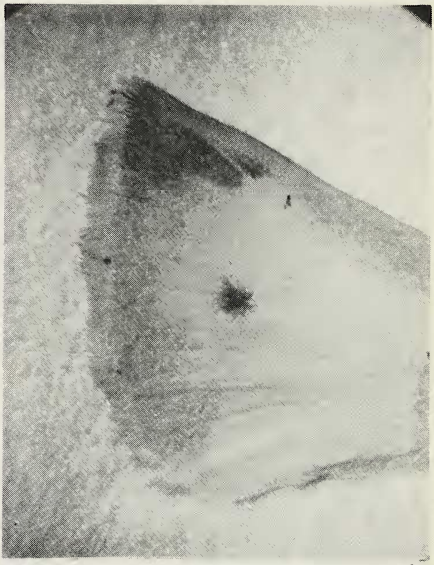

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PLATE I

Fig. 1 (upper left) — Upper spot of wing from pupa (♀) about 175 hours old, showing "relief" effect. The illuminating light beam was 10° from the horizontal to give shadowing.

Fig. 2 (upper right) — Upper spot of wing from pupa (♀) about 195 hours old ,with markings in early stage of darkening. Lighting as in Fig. 1.

Fig. 3 (lower left) — Artificial pigmentation of wing from 190 hour old pupa (♂) by aqueous extract of hemolymph (details in text).

Fig. 4 (lower right) — Artificial pigmentation of wing from 190 hour old pupa (♂) by undiluted hemolymph (details in text).

The object of the present work was to study pattern development for *Pieris rapae* (L.) in order to contribute original observations as well as to evaluate the explanations of Onslow and Braun.

EXPERIMENTAL

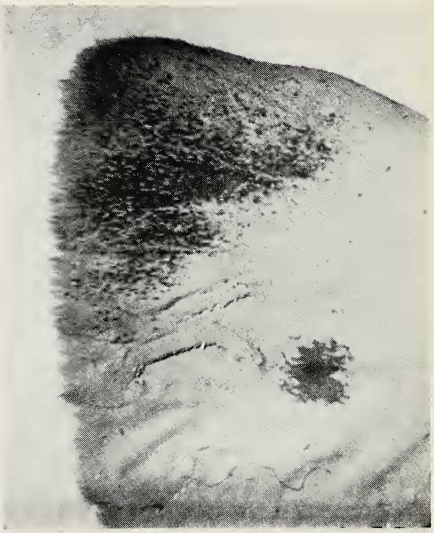
Source of pupae — Final instar larvae were supplied by the U.S. Department of Agriculture (see Acknowledgement). These had been reared on an artificial diet at 26 ± 1°C and 45 ± 5% relative humidity under continuous cool white fluorescent light. Development was completed on cabbage leaves from refrigerated heads. Pupae were kept in a room at 70-80°F and 28-35% relative humidity.

Dissection techniques. — Early pupae, e.g. 90 hours old, presented more of a problem in removal of the forewing than did pupae at later stages, after the wingcases had whitened at about 135 hours. However, a successful procedure was to cut off the head end of the pupa, just at the base of the wings, by pushing downward with a razor blade. Then a small pair of scissors was used to cut all around the wingcase. The wingcase was placed on a table inside up, a small piece of blotting paper was used to remove matter (including the hindwing) covering the forewing, the nail of the left index finger was applied to hold down the basal end of the integument, and the wing was grasped at the base with pointed forceps and carefully peeled off to be placed in water or aqueous solution.

As the time of eclosion drew near, it became possible to dissect out the forewings merely by cutting the pupal case and pulling the wing out by the base.

The wings shown in Plates I-III were allowed to dry in air, and each was mounted on a microslide on a square of white blotting paper under a cover glass, the latter being held in place by a gummed label with appropriate hole. The photographs were made through a 100X microscope for Figures 1 and 2, a 1000X microscope for Figure 8, and a 16X microscope for the other Figures.

Observations. — Most of the observations were made with a stereo microscope at 16X. Illumination was a concentrated spot of light from a microscope illuminator aimed down on the subject at a 45° angle. Features of the wing such as venation and areas of translucent scales were seen most clearly against a background of black felt, but judgment of degree of darkening



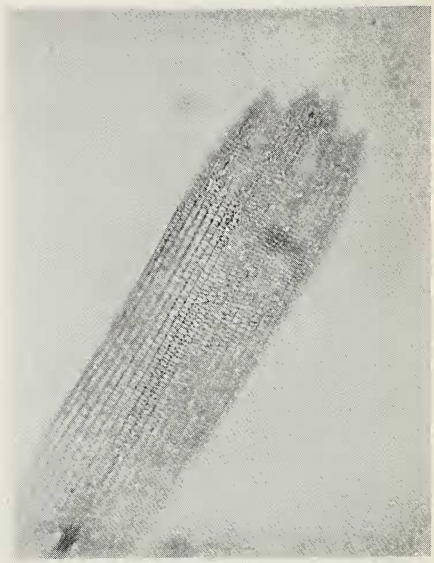


PLATE II

Fig. 5 (upper left) — Wing from 160 hour old pupa blackened by exposure to dopa solution followed by drying in air (details in text).
Fig. 6 (upper right) — Artificial pigmentation of wing from 185 hour old pupa (♀) by dopa solution (details in text).
Fig. 7 (lower left) — Control wing (in water) for wing shown in Fig. 6.
Fig. 8 (lower right) — Dark scale from the spot of a wing from a 175 hour old pupa (♀), artificially pigmented by dopa solution (details in text). The scale was mounted in Permount (Fisher Scientific Co.) and photographed by transmitted light through a microscope with 10X wide-field ocular and 100X achromatic objective (1.25 N.A., oil immersion).

of scales was made against a white background. Close observation of the scales was made with a biological microscope at 100X or 430X with either reflected or transmitted light or at 1000X (oil immersion) with transmitted light.

Wings were soaked conveniently in solutions in uncovered watchglasses at room temperature (70-80°F) for a few hours, but longer times required closed containers to prevent evaporation.

Solutions and reagents.—

Saturated tyrosine solution: Excess L-tyrosine (Matheson Coleman and Bell) was shaken with deionized water. The concentration is reported to be 0.045% at 25°C (Anonymous, 1960).

Dopa solution (0.5%): In 20 ml deionized water was dissolved 0.10 grams of DL-3-(3,4-dihydroxyphenyl)alanine (practical grade, Matheson Coleman and Bell). The solubility of the less-soluble L-form is 0.5% (Anonymous, 1960). A 0.4% solution was used by Gonnard and Svináreff (1951) as substrate for potato tyrosinase.

Iodine reagent: According to the method of Campbell (1929), a solution of 1.2 grams iodine and 1.6 grams potassium iodide in 1.5 ml water was added to 50 grams of 20% acetic acid to give a clear, dark-red solution.

Tollen's reagent: Small portions were prepared according to Feigl (1954) and used immediately (the solution cannot be stored as it decomposes and deposits explosive silver fulminate). A convenient amount (about 0.6 ml) was given by adding 5 drops 10% sodium hydroxide to 5 drops 10% silver nitrate to give a brown precipitate and then dissolving this by addition of 3 drops of a mixture of equal volumes conc. ammonium hydroxide (28-30% NH_3) and water.

Le Rosen formalin reagent: Since the reagent cannot be stored, a small volume was prepared just before using by stirring 2 drops of 37% formaldehyde solution into 10 drops conc. (98%) sulfuric acid in a watchglass. This is a variation (higher formalin content) on the reagent according to Feigl (1954).

Misc. solutions: Concentrations are given in weight-%, e.g. 50% sulfuric acid was prepared by adding 50 grams acid to 50 grams water. The water used was always distilled and then deionized. 4-Chlororesorcinol (Koppers Co.) was recrystallized to give capillary melting point 108.5-110°C. The other organic compounds were used as supplied by Matheson Coleman and Bell or Eastman Organic Chemicals.

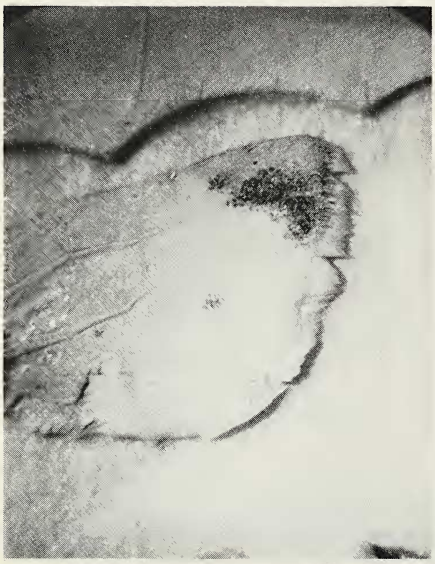


PLATE III

Fig. 9 (upper left) — Wing from 175 hour old pupa (♀) soaked in oxygen-free dopa solution (no pigmentation occurred) and then allowed to dry in air (markings darkened; details in text).

Fig. 10 (upper right) — Wing from 189 hour old pupa (♂) tinted deep pink by murexide formation (details in text). The spot (pale yellow) is faintly visible in the photograph.

Fig. 11 (lower left) — Wing dissected from pupa (♂) with markings just beginning to darken; shown after 18 hours in water-saturated air.

Fig. 12 (lower right) — Control wing (18 hours in water-saturated nitrogen) for the wing shown in Fig. 11.

RESULTS AND DISCUSSION

1. Structural Changes During Development

Chronology of development. — The following notes on scale development are preliminary observations based on a few dissections. Times from pupation (final larval molt to give pupa) are approximate and intended to be typical; some pupae developed more slowly.

27 hours: Careful dissection gave a tracheated wing with frothy appearance at 430X. This seemed to consist of epithelium with scales not yet grown. The wing epithelium of the freshly-molted *Pieris brassicae* pupa is composed exclusively of stem cells which later give the scale and socket arrangement (Lipp, 1957).

87 hours: Scales covered the wing. These were round, generally about 0.02 mm in diameter but some larger.

101 hours: Scales were generally round, approx. 0.04-0.09 mm in diameter. Staining with 1% crystal violet (Colour Index No. 42555) in 95% ethanol or with a 1:1 mixture of saturated safranin O (Colour Index No. 50240) in 95% ethanol with aniline water (Shillaber, 1944) helped make the scales visible.

122 hours: Hairlike fringe scales on the margin were conspicuous against a black background. Scales on the wing were of various shapes, some round, usually with a point, others somewhat elongated with three teeth. Length varied from about 0.04 to 0.14 mm. The impression was that some of the round scales grow into the elongated form, length about 0.13-0.17 mm, that is most common on the adult wing. Exposure to tyrosine or dopa solutions darkened the scales and improved visibility at 100 or 430X.

135 hours: The wingcases became noticeably whitened to the naked eye. Also noted was disappearance of the former translucency of the pupa in the wing region when viewed from the side against a source of light.

165 hours: The future markings (spots), though not at all pigmented, were dimly visible through the pupal case.

175 hours: Female wings had a yellow appearance as viewed through the pupal case.

195 hours: Darkening of the markings began. The most slowly developing pupae reached this stage in 220 hours.

200 hours: Markings were completely darkened. The darkening process required 4-5 hours at 80°F. The wings shortly later became hydrophobic whereas in earlier pupae they were

easily wetted when dissected and placed in water.

220 hours: Eclosion.

Ages of pupae given below were adjusted in some cases in order to indicate point of development in terms of the above schedule.

Visible distinction of future-black scales. — The future-black scales in the apical area and, particularly, in the spot(s) (one on male, two on female wing) became noticeably different in appearance (glossy by reflected light at a certain angle, translucent by transmitted light) at about 135 hours. A good description is "like spots of grease upon white paper" (Onslow, 1916). At 155 hours the scales all collapsed against the membrane when the wing was dried in air, but at a later time, e.g. 175 hours, a "relief stage" (Braun, 1939) became obvious after air-drying for only a few minutes. With side-lighting, the spot scales appeared to have collapsed against the wing membrane while the surrounding scales remained erected. An example is shown in Plate I, Figure 1. This effect was no longer well-defined on a pupal wing with markings just beginning to darken (Plate I, Figure 2).

2. Artificial Pigmentation

Water and saline. — In no case among the many forewings exposed to water did darkening occur in times up to 6 hours, but at 37 hours (pupa 173 hours old) there was darkening (brownish color) at the base and very slight darkening of the future-black scales, the rest of the scales remaining the original white. The other wing of the 173 hour old pupa was exposed to 0.05 M sodium chloride for 37 hours with no darkening. However, a wing from a 190 hour old pupa showed darkening at the torn base in 0.05 M sodium chloride at 9 hours, and the spot was very pale brown (wing itself very pale yellow-tan) at 48 hours. This NaCl concentration is in the general vicinity of the chloride content of the pupal blood, e.g. 0.02 M in chloride for the *Pieris brassicae* pupa (Buck, 1953). Onslow (1916) reported slight darkening of markings on the pupal wing of *P. brassicae* after 12 hours in "normal saline" (0.75% NaCl).

Hemolymph. — Hemolymph, as obtained in diluted form by grinding pupae with chloroform water and filtering, was reported by Onslow (1916) to give considerable darkening of the markings of the pupal wing of *P. brassicae* in 12 hours.

In the present work, diluted hemolymph was prepared from a 190 hour old pupa by grinding all but the forewings with 1.5

ml deionized water and filtering to give a colorless, opalescent liquid, in which one forewing was placed. The future-black scales were very pale brown after 33 hours vs. no appreciable darkening for the other (control) wing in deionized water. In another experiment, the wings were removed from a 190 hour old pupa, and the remainder of the pupa along with three pupae with markings darkened was ground with 6 ml water (pH 5.6) and filtered. Part of the filtrate (pH 6.6) was added to one wing, and the remainder was adjusted to pH 8.0 with several drops of 0.1% sodium carbonate solution and added to the other wing. The same procedure then was repeated using chloroform-saturated water. The result was that both water and chloroform water extracts gave light-brown future-black scales visible against the pale-tan future-white scales, but the water extract seemed to give slightly more darkening (Plate I, Figure 3). Results at pH 8.0 were not so good as at pH 6.6, especially for the chloroform water (negligible darkening of future-black scales). The pH of *P. rapae* pupal blood has been reported as 5.9-6.4 (Buck, 1953).

A drop of clear, pale-green hemolymph was noted to exude from the body of a 215 hour old pupa (markings fully darkened) from which the head end had been cut at the base of the wings. This liquid was placed on one wing from a 190 hour old pupa, and the other wing was placed in water as a control. After 5 hours some darkening of the markings of the wing with hemolymph was noted, and a small amount of water was added to prevent desiccation. At 10 hours the markings were well darkened in the hemolymph case (Plate I, Figure 4) vs. no darkening of future-black scales for the control wing. The contrast between markings and white scales was more pronounced (white scales less darkened) for the wing shown in Figure 4 than in artificial pigmentations with dopa solution.

Tyrosine. — Saturated tyrosine solution caused rapid blackening (in less than 30 minutes) at the edge of the torn base of the wing, as did 0.5% dopa, presumably because of the tyrosinase-containing hemolymph exposed in this area. For a 165 hour old pupa the markings (spot and apex) darkened slowly; the scales within the spot were pale gray after 6 hours. As a control, the other forewing from the same pupa was soaked in deionized water and showed no darkening after 6 hours.

Braun (1939) claimed that wings in tyrosine solution unfolded (expanded), a phenomenon produced by "no other solution tested". In the present work there was much individual variation in the extent of expansion, but all the aqueous chromogen solu-

tions, 0.05 M NaCl, and deionized water itself gave this effect. Using the distance from apex to outer angle (4-5 mm for untreated pupal wing, typically 14 mm in adult) as a measure of expansion, the following values were noted for pairs of wings from the same pupa: 11 mm for saturated tyrosine solution vs. 9 mm for water, 6.5 mm for 0.5% dopa solution vs. 7 mm for water, and 10 mm for 0.1% sodium carbonate solution vs. 12.5 mm for water (the greatest expansion noted). Pupal age may have a large influence on degree of expansion. Tyrosine solution obviously is not unique in causing expansion, and possible marginal superiority over water or other aqueous solutions would have to be demonstrated by a number of competitive experiments.

Dopa. — The use of tyrosine solution soon was discontinued in favor of 0.5% dopa, since the latter was found to be far superior for artificial pigmentation. For example, at 6 hours, one wing (from 124 hour old pupa) in dopa solution was well darkened (deep gray) while the other wing in tyrosine solution had only a light gray cast. This result would be expected because dopa is an intermediate between tyrosine and melanin (oxidation of tyrosine to dopa by tyrosinase is easily demonstrated — Evans and Raper, 1937), dopa is more sensitive to enzymic oxidation than many other chromogens (Schmalfuss, 1924), and dopa is even readily oxidized nonenzymically, e.g. the 0.5% solution begins to turn brown in a few days.

At 131 hours pupal age, dopa solution caused the whole wing to become light gray in 3.5 hours with no selective darkening of the future-black scales. At 160 hours pupal age, dopa solution after 1.5 hours caused a wing to become gray with no differentiation (except translucency) of future-black scales; after rinsing with water and air-drying overnight the wing was dark gray, almost black, with markings barely discernible (Plate II, Figure 5). At about 185 hours pupal age, exposure to dopa solution gave selective darkening that remained clear after the wing had been rinsed with water and air-dried (Plate II, Figure 6). The other (control) wing in deionized water did not darken (Plate II, Figure 7). Figure 8 (Plate II) shows a dark scale from the spot on a female wing (from 175 hour old pupa) which had been artificially pigmented in dopa solution for 1.5 hours. Minute spots of pigment are visible, seemingly within the sub-

stance of the scale as claimed by Onslow (1916), Reichelt (1925), and Braun (1939).

When exposure to dopa solution was continued, for a pupa about 190 hours old, the spot and apical scales were black against a dark gray background at 24 hours, and at 48 hours the wing was very dark gray, almost black, with markings barely discernible (resembling Figure 5 n Plate II). The white scales on the wing of a 204 hour old pupa, with markings recently darkened, became very light gray after 4.5 hours in dopa solution, and an even older pupa, apparently ready to eclose, gave the same result.

The indication is that in earlier stages, e.g. 160 hours old or less, all scales became pigmented at the same rate, while later on (185 hours old or more) the future-black scales darkened sooner but were eventually nearly equalled by the slower-darkening future-white scales. Artificial pigmentation with dopa thus is a "kinetic effect" resulting from the slower rate of darkening of the future-white scales, not their inability to darken.

Other chromogens. — Cresols (54% m-, 29% p-, 17% other phenols), DL-beta-phenylalanine, p-aminopehnol, resorcinol, and catechol were tested as 0.5% solutions with the other wing from each pupa (about 170 hours old) in 0.5% dopa solution. At 3 hours, all the wings in dopa solution were gray with future-black scales darker gray. Phenylalanine and resorcinol gave no darkening, the cresol mixture gave an orange tint to the basal half of the wing but no darkening of future-black scales, p-aminophenol gave a tan-gray tint to the whole wing with doubtful darkening of the markings (translucency was difficult to distinguish from pigmentation), and catechol gave an overall orange-gray color with future-black scales darkened. These results agree with the literature. The tyrosinase of the *P. rapae* pupa oxidized catechol more readily than p-cresol (Pugh, 1934). Tyrosinase from the meal worm oxidized p-aminophenol but not resorcinol (Gortner, 1910).

Inhibition by chemicals. — Melanogenesis inhibitors (see Kolyer, 1966) were tested by adding at 0.5% to a 0.5% dopa solution, with the other wing of each pupa (about 174 hours old) in 0.5% dopa solution as a control (all turned gray with markings very dark gray in 3 hours). Thiourea and L(+)ascorbic acid allowed no darkening of wing or markings, while the wing became light gray but with little darkening of the markings with hydroquinone or 4-chlororesorcinol. Thiourea has been shown to

cause pronounced inhibition of phenoloxidase activity in silkworm homogenates (Chmurzynska and Lech, 1963) and is a well-known melanogenesis inhibitor. Ascorboic acid is a melanogenesis inhibitor in vitro but is considered necessary in the diet for optimum development of the silkworm (Ito, 1961). None of these inhibitors prevented pigmentation when fed to larvae in earlier work with *P. rapae* (Kolyer, 1966).

When the test was repeated (pupae about 177 hours old) with the inhibitors (except hydroquinone) at 0.05%, i.e. 10% on the level of dopa instead of 100% as in the first test, the result was partial inhibition (markings darkened but less intensely than in the controls). Using pupae at about 193 hours old, at 0.005% inhibitor (1% of dopa level) there was little, if any, inhibition. Thiourea (at 0.5%) also inhibited darkening of the markings (pupa about 193 hours old) in 0.5% catechol solution for 3 hours, but the wing became pale orange-gray overall.

Inhibition by heat. — Gortner (1910, 1911b) reported that activity of tyrosinase from the meal worm or the periodical cicada is destroyed by heating at 75°C for one minute, and Onslow (1916) found that boiling the pupal wing of *P. brassicae* prevented darkening in tyrosine solution. This denaturation of the enzyme by heat was confimed for *P. rapae* as follows. One wing from a 190 hour old pupa was placed in 1 ml water in a small test tube, which then was immersed in water at 88-90°C for 5 minutes. After 48 hours in dopa solution the wing was pale yellow with no darkening of the future-black scales, while the other (unheated) wing showed darkening of the future-black scales in 2 hours.

In a series of hemolymph tests, the body fluid was squeezed from three pupae (160 hours old) into a micro test tube, which then was heated in water at 88-90°C for 5 minutes. The resulting semisolid paste was diluted with 0.09 ml water and filtered by drawing into a pipet plugged with cotton wool, and the filtrate was applied to heated and unheated wings. Unheated, filtered fluid similarly was applied to heated and unheated wings. At 48 hours, the unheated wing with unheated hemolymph was pale tan with darkening at the torn base but no darkening of the markings. The unheated wing with heated hemolymph was gray-brown with darkening of the apex but not the spot. The heated wing with unheated hemolymph was tan with markings not darkened. The heated wing with heated hemolymph was pale orange-yellow with no darkening. Though the markings

in this series were not darkened when both hemolymph and wing were unheated, as they were in other experiments, general darkening of the wing was prevented only by heating *both* wing and hemolymph. This is in agreement with the data of Onslow (1916) and is explained by the presence of both oxidase and chromogen in the wing as well as in the hemolymph.

Role of oxygen. — The hemolymph, which contains both oxidase and chromogen, darkens when contacted with the atmosphere (Pugh, 1934, and Wigglesworth, 1965: 383). In the present work it was noted that darkening proceeded within a minute when pupal hemolymph diluted with water was sparged with fine bubbles of air.

In the enzymic oxidation of dopa, oxygen is necessary, though only a trace is required (Gortner, 1911a, and Schmalfuss, 1924). That dissolved oxygen was essential for the artificial pigmentations described above was indicated by soaking a wing (from 175 hour old pupa) in a 0.5% solution of dopa in essentially oxygen-free deionized water (prepared by boiling 10 minutes and cooling while bubbling in high-purity nitrogen). After 2 hours there was no darkening, even at the torn base, while the other wing from the same pupa in ordinary (air-containing) 0.5% dopa solution was blackened at the base and had the future-black scales gray. The oxygen-free wing was rinsed with water and allowed to dry in the air for 1.5 hours, during which time artificial pigmentation proceeded, presumably due to availability of oxygen (see Plate III, Figure 9).

3. Chemical Tests

Iodine solution. — Braun (1939) utilized a test (treatment with iodine solution, then zinc chloride solution) said to distinguish between "hard" and "soft" chitin and found for *Papilio ajax* and *Ephestia kühnella* that future-dark scales appeared light and future-white scales appeared dark. This result, according to Braun, "exhibits clearly that the different parts are found in different stages of chitinization". However, the test used by Braun in not necessarily a specific test for chitin (Richards, 1947). Also, the hardest cuticles often contain less chitin than the soft (Wigglesworth, 1965: 32). Richards (1947) treated *P. rapae* scales with hot alkali and applied the chitosan test (Campbell, 1929), which probably proves the presence of chitin when it is positive, as it was for the dark scales. The white scales were dissolved, but chitin in some cases is destroyed by hot alkali, so that the presence of chitin in the white scales could not be discounted.

In the present work, pupal wings were treated with iodine solution (see Experimental) as used in the chitosan test (Campbell, 1929). Chitin itself (Matheson Coleman and Bell practical grade, prepared by purifying crab shells) was stained dark brown by this reagent. For wings of pupae about 175 hours old the spot was orange against a pale orange background after iodine treatment, but translucency was a factor in this appearance. Addition of 10% zinc chloride gave no color change, but addition of 50% sulfuric acid caused rapid darkening of the wing with, in some cases, marked resistance to darkening by the spot so that it appeared as a "window". This may be the result of absence of pterin pigments in the future-black scales, because particles of leucopterin (from K & K Laboratories, Inc.) were stained brown by treatment with the iodine solution when followed by 50% sulfuric acid but not when followed by 10% zinc chloride. Thus, pterin pigment distribution rather than differences in chitinization possibly may explain the selective staining reaction described.

Murexide test. — The forewings from a 189 hour old pupa were exposed to chlorine gas for 9 hours and left in the air for 17 days. The wings, pink only around the edges, then were exposed to gaseous NH_3 for 5 minutes, during which time they became deep pink (rose color). The spots remained pale yellow against the pink background (see Plate III, Figure 10), presumably because the future-black scales were free of pterins such as leucopterin, xanthopterin, and isoxanthopterin, which give the murexide color (Ford, 1947, and Gates, 1947). Leucopterin, isoxanthopterin, and other pterins have been found in the *P. brassicae* pupa (Busnel and Drilhon, 1949, and Harmsen, 1966).

Ammoniacal silver nitrate. — The argentaffin reaction is used to identify o-hydroxyphenols such as dopa (Richards, 1953), which reduce the reagent to give free silver (black). Various solutions have been described, all containing the readily-reduced complex of silver ion with ammonia or amines. In the present work Tollen's reagent was found convenient.

Wings from 123, 125, and 131 hour old pupae were covered with fresh Tollen's reagent and within 5 minutes had turned faint brown with no differentiation of the spot (which does not become visible by translucency until about 135 hours). However, a wing from a female pupa about 175 hours old began to darken immediately and in 2 minutes was practically black with the two spots appearing as colorless "windows". The other

wing was soaked in water for 15 minutes before adding the reagent; the result was the same except that the wing darkened less (to brown rather than almost black). The wing from a 204 hour old pupa, with markings darkened, turned dark gray after 2 minutes in the reagent so that the markings were barely discernible. The same result was given by an adult wing (16 months old).

Selected white flakes of chitin were pale gray after 3 minutes in the reagent, while particles of leucopterin turned brown to black within 2 minutes. Urates, which are somewhat similar chemically to the ammonium salt of leucopterin presumably formed on adding Tollen's reagent, are said to give a positive argentaffin test (Richards, 1951: 71). Isoxanthopterin reduces Tollen's reagent (Gates, 1947).

Paper chromatography was done to verify the presence of pterins in the pupal wing. Extracts were prepared from adult wings (mixed sexes, washed with ether) or pupal forewings (separate extracts for male and female wings from pupae about 190 hours old) by soaking the wings in 20% ammonia solution for a few hours. The extracts were chromatographed vs. a solution of leucopterin in 20% ammonia on Whatman No. 40 filter paper by the ascending method (40 minutes at 78°F; solvent front ran about 64 mm above point of application of extracts). The solvent system was that of Partridge (1948) as recommended for pterins by Good and Johnson (1949). This was prepared by shaking 40 ml n-butanol, 10 ml acetic acid, and 50 ml water, allowing to stand 4 hours, and discarding the lower (aqueous) layer. After drying, the paper was viewed under ultraviolet light (mainly about 360 millimicrons). The adult wing extract gave two fluorescent spots: very pale blue, R 0.11 (Good and Johnson report 0.12 for leucopterin), and bright purple, R 0.28 (Watt and Bowden (1966) report 0.24 for isoxanthopterin). These same two spots have been reported for adult wings of *Pieris rapae*, *P. brassicae* and *P. napi* (L.). The pupal wings also showed these two spots as well as an additional spot, pale yellow, R 0.39, which was more intense in the female wing; Good and Johnson assign this (R 0.38) to xanthopterin. Tollen's reagent poured over the paper caused intense darkening of the spots of application of both the wing extract and the leucopterin, indicating that much of the leucopterin applied to the paper failed to migrate with the solvent.

The tentative conclusion is that the positive Tollen's test is caused by pterins and urates associated with the future-white

scales. The weaker test after exposure of the wing to water may have been due to partial extraction of pigment materials.

Formalin-sulfuric acid. — Le Rosen formalin reagent added to wings from pupae about 190 or 198 hours old caused red-violet staining along the veins to a distance of about half way out on the wing, which appeared clear pale-yellow. A fragment of muscle tissue from the thorax showed red-violet streaks when treated with the reagent. Crystals of dopa dropped in the reagent also gave a red-violet color, as has been reported (Denigès, 1926). Catechol gave the same result, but L-tyrosine dissolved and then reappeared as a white precipitate, presumably the sulfate, without giving a color. A red-violet color is said to be given by phenols in general (Feigl, 1954). In the present instance the color is attributed to phenolic substances, such as dopa, in the hemolymph of the veins. No violet color in the region of the markings was observed. An interesting incidental effect was the clear display of the tracheae due to transparency afforded by the reagent.

Concentrated sulfuric acid, without formalin, gave no color, nor was rapid dissolution of the scales observed as described by Braun (1939). Incidentally, a technique for isolating the wing membrane was provided by exposing the wing to the acid for 5 minutes followed by a water rinse. It was then easy to push away the scales as a soft mass.

4. Natural Pigmentation

Some experiments were performed to evaluate the suggestion of Onslow (1916) that pigmentation is triggered by exposure of the wing surface to air. When pupae with markings just starting to darken, or even half-darkened, were placed in nitrogen, either dry or saturated with water vapor, pigmentation was arrested. Results with isolated forewings were consistent. The forewings were dissected from a female pupa with markings judged just about to darken and placed in separate vials, one filled with nitrogen and the other with air. A drop of water was present in each case to saturate the gas and prevent desiccation. After 7 hours the markings of the wing in air were about half darkened, while no darkening had occurred in nitrogen. In a similar experiment, male forewings, originally slightly darkened, were left in humid air vs. nitrogen for 18 hours. The result was further darkening in air vs. no change in nitrogen; see Plate III, Figures 11 and 12.

The pupal case was removed from one wing of a pupa with markings half darkened. The imago eclosed 14 hours later with markings fully darkened on the wing that had remained covered but arrested at half darkened on the wing that had been exposed. In a similar experiment, the markings were just starting to darken when one wing was exposed. Again, the markings on the exposed wing failed to darken, while the markings on the covered wing were perhaps 75% darkened (in terms of final intensity) after 4 hours.

Since the above results were attributed to desiccation by evaporation of water from the exposed wing, pupae with the apex of the wing exposed were placed in vials containing cotton wool saturated with water to provide 100% relative humidity. For air, results (initiation of darkening, completion of darkening, eclosion), in hours from start of experiment, were: 13, 20, 25; 1, 5, 15; 0, 5, 19. For oxygen, results were: 19, 24, 36; 0, 7, 13. In all cases there was no difference at any time in appearance of the exposed vs. the covered apex.

Using two pupae with markings not yet starting to darken, the apex of one wing was exposed and covered with petroleum jelly in an attempt to exclude air from the wing surface. Approximate times (in hours as above) were: 31, 35, 38, and 22, 27, 31. In the first case no difference was observed during pigmentation of the apices, but in the second case there was a delay in pigmentation of the outer part of the petrolatum-covered apical marking. The fact that a delay was observed suggests that contact of the scales with air is a requirement, but the data of the preceding paragraph show that the apex can be directly exposed to air for 13 hours (or to oxygen for 19 hours) before pigmentation commences.

CONCLUSION

Artificial pigmentation. — Oxidase must exist in both future-black and future-white scales, since both eventually darken when chromogen, e.g. dopa, is supplied. But why is artificial pigmentation much more rapid for the future-black scales? Braun (1939) argued that these scales are "softer" and "less chitinized" but failed to prove chitinization of the future-white scales. Even if selective chitinization were demonstrated, the literature indicates this would not necessarily mean greater hardness and lower permeability. Alternative possibilities to greater permeability of the future- black scales are (1) less oxidase in the future-white scales and/or (2) inhibition of

melanogenesis in the future-white scales. The latter idea may have merit on the basis that considerable evidence was given by the chemical tests for the presence of pterins in the future-white scales and the substantial lack of these pigments in the future-black scales, and leucopterin, xanthopterin, and isoxanthopterin have been shown to have an inhibitory effect on potato tyrosinase *in vitro* (Gonnard and Svináreff, 1951, and Isaka, 1952).

The "relief stage" effect seems to indicate greater rigidity for the future-white scales, but there is no reason to assign this to "chitinization." Hardening of the protein of the walls of the scale, without chitin, seems as good a supposition since Richards (1947) failed to demonstrate chitin in the white scales of the adult *P. rapae*. It even seems possible that the white pigment itself could have a reinforcing effect by being deposited in the striations or corrugations of the scale.

In fact, the appearance of pigment at about 135 hours in the future-white scales might explain all the observations. The question then would revert to — What causes this selectivity of deposition of pterin pigments?

Whatever the explanation, artificial pigmentation seems only a relatively pale and less selective simulation of the natural process in which some scales remain pure white while others blacken intensely.

Natural pigmentation. — Braun states: "At a certain time in development pigment is present in the body and the subsequent dark parts, being still soft at this time, will deposit pigment". Presumably by "pigment" is meant chromogen. Also: "If at a certain time in the development tyrosine is present, it will only be deposited in those scales which represent a certain condition of the chitin at this moment, which means only a certain part of the pattern". However, tyrosine is found freely in insect blood (Brunet, 1963) and was found in the larvae and pupae of *Pieris brassicae* (Stamm and Aguirre, 1955) and of the silkworm (Watanabe, 1956a, and Tomino, 1963 and 1965). Dopa itself has been suggested as the chromogen in *P. rapae* (Goodwin, 1965) and is present in all stages of the silkworm (Watanabe, 1956a and b). Thus, the chromogens tyrosine and dopa seem to be present at all times. Furthermore, according to Buck (1953), "There is reason to believe that enzyme, substrate, and adequate oxygen are present together in the blood for some time prior to the actual formation of pigment. The puzzle, therefore,

is not so much in how melanin is formed, but in how its formation in the blood of the intact animal is prevented and its formation in cuticle so narrowly limited in time".

The well-documented necessity for oxygen in natural pigmentation was verified, and direct contact with the scales seems to be required, which is consistent with the low capacity of hemolymph to transport oxygen (Buck, 1953). However, Onslow's suggestion that pigmentation is triggered by air becoming available due to pulling away of the wing from the pupal case was discounted by removing a section of the pupal integument at the apical region and finding delays of up to 13 hours in air or 19 hours in oxygen before pigmentation commenced. An interesting example of oxygen supply as necessary but not sufficient to initiate pigmentation is given by Fraenkel (1935) for the newly eclosed blow-fly *Calliphora erythrocephala*. Pigmentation was inhibited and postponed by allowing the flies to dig for an abnormally long time through sawdust in the presence of air, showing that exposure to oxygen on emergence from the pupal case was not sufficient to cause chromogen to oxidize but that there is "certainly a nervous mechanism involved in initiation of the coloraton process". This nervous mechanism might function through a shift in oxidation-reduction potential of the blood due to stress; see Buck, 1953.

In concluson, the complexity of the living system, both structurally and chemically, makes dubious any simplistic mechanism that might be proposed to explain pigmentation. Some points can be demonstrated, but no general hypothesis, e.g. that of Braun (1939), is very convincing when alternate explanations can be suggested which also fit the limited data.

SUMMARY

Pupal wings of *Pieris rapae* (L.) were dissected and studied at various times from pupation to eclosion (9-10 days). Scales grew to full size from approximately 3 to 5 days with no apparent difference between the future-white and future-black varieties. At 5-6 days the wingcases became noticeably whitened. During the next 3 days, before the onset of black pigmentation, the presence of pterin pigments in the future-white scales, and their substantial absence in the future-black scales, was indicated by dark staining of the future-white scales with iodine solution followed by 50% sulfuric acid, selective reduction of ammoniacal silver nitrate by the future-white scales, and selec-

tive pink coloration of the future-white scales by the murexide test (chlorine treatment). Also, the future-black scales, lacking pigment, were relatively translucent. A "relief stage", as reported by W. Braun (1939) for species including *Papilio ajax* (L.), was seen on brief drying of the wing in air; the future-white scales appeared erect, the future-black scales collapsed. Artificial pigmentation, reported by Braun using saturated tyrosine solution, was more effectively achieved with dopa. Another successful chromogen was catechol. Before white pigmentation at 5-6 days, all scales darkened in 0.5% DL-dopa solution at the same rate, but in older pupae the future-black scales darkened faster and so were blackened selectively at short times, e.g. 2 hours, though the whole wing became very dark by 48 hours. This process is an enzymic oxidation requiring traces of oxygen and prevented by melanogenesis inhibitors such as thiourea or ascorbic acid or by brief heating of the wing at 90°C to destroy the oxidase. Premature pigmentation also was achieved by soaking the wing in pupal hemolymph, whereas darkening of the future-black scales in water was at best faint. Thus, the data indicate that the scales contain oxidase but are deficient in chromogen. The reason for the pronounced difference in rate of darkening in dopa solution between future-black and future-white scales was not clear. Alternative explanations include less oxidase in the future-white scales, greater permeability of the future-black scales, and inhibition of melanin formation in the future-white scales. The selective presence of pterin pigments in the future-white scales possibly might explain not only artificial pigmentation (pterins are known melanogenesis inhibitors) but also the "relief stage" by reinforcing effect of pigment deposited in the walls of the scale. The complexity of this biological system, and the variety of explanations fitting the limited data, make questionable the simplistic explanations that have been proposed for black pigmentation *in vivo,* of which artificial pigmentation is a pale and relatively nonselective simulation. Pigmentation *in vivo* is not triggered by exposure of the wing surface to air, as has been suggested (Onslow, 1916), because darkening commenced and proceeded normally at times of up to 19 hours after removal of pupal integument to expose the apex of the wing to water-saturated air or oxygen. This result, coupled with the observation of a delay in darkening vs. the untreated apex when the exposed apex was covered with petroleum jelly to exclude air, suggests that availability

of oxygen at the wing surface is necessary but not sufficient to initiate the rapid (about 5 hours at 80°F) formation of black pigment.

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